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Purification of a fucoidan from kelp polysaccharide and its inhibitory kinetics for tyrosinase

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ABSTRACT

High-speed countercurrent chromatography (HSCCC) was used to separate kelp polysaccharide. HSCCC was performed using an aqueous two-phase solvent system composed of PEG1000–K_2HPO_4–KH_2PO_4–H_2O (0.5:1.25:1.25:7.0, w/w) by eluting a lower aqueous phase at 2.0 mL/min at 600 rpm, yielding two separate fractions, KPS-1 and KPS-2. The KPS-2 fraction was further purified by DEAE-Sepharose fast flow anion-exchange column chromatography to provide 3 fractions, KPS-2-1, KPS-2-2 and KPS-2-3. GPC–HPLC analysis indicated that KPS-2-1 fraction was a purified fucoidan. FT-IR analysis showed that KPS-2-1 was a sulphated polysaccharide. An analysis of enzymatic kinetics showed that the purified fucoidan was a competitive inhibitor of tyrosinase toward L-tyrosine, and the inhibitory constant K_i obtained from double-reciprocal plots was 0.9907 mg/mL.

1. Introduction

Fucoidan is a complex sulphated polysaccharide that is mainly derived from marine brown seaweed. Usually, fucoidan contains large proportions of L-fructose and sulphate (Bilan, Grachev, Shashkov, Nifantiev, & Usov, 2006; Duarte, Cardoso, Noseda, & Cerezo, 2001). Previous studies have shown that fucoidan has a wide variety of bioactivities, such as inflammatory modulation and anti-virus and anti-tumor activities (Alekseyenko et al., 2007; Chandía & Matsuhiro, 2008; Cumashi et al., 2007; Hayashi, Nakano, Hashimoto, Kanekiyo, & Hayashi, 2008; Yang, Aisa, & Ito, 2009). Recently, a fucoidan from algae *Fucus vesiculosus* has been demonstrated to inhibit human immunodeficiency virus (HIV) *in vitro* and shows a synergistic effect with azidothymidine (Chotigeat, Tongsupa, Supamataya, & Phongdara, 2004; Sugawara, Itoh, Kimura, Mori, & Shimada, 1989). Brown seaweed kelp (*Laminaria japonica*) is one of the most important economic seaweeds cultured in China, Japan and Korea, and is widely consumed as a marine vegetable in these countries (Gao, Qin, & Zhang, 2006; Pang, 2007; Suzuki, Furuya, & Takeuchi, 2006). The use of kelp as a drug and an effective component of cosmetics have been well documented in traditional Chinese medicine (Zhang et al., 2007). Kelp is also used as manure, cattle feed, and food for human consumption, as well as a source of phycocolloids, such as agar, alginic acid and carrageenan (Chapman, 1970).

High-speed countercurrent chromatography (HSCCC) has been widely used for the separation and purification of natural products (Yang et al., 2009). HSCCC is a liquid chromatographic technique that operates under gentle conditions and allows non-destructive isolation, even for labile natural compounds. Due to the absence of any solid stationary phase, adsorptive losses are minimized, guaranteeing a 100% sample recovery (Scharnhop & Winterhalter, 2009). To date, the use of HSCCC to isolate products has focused on small organic compounds, and very few efforts have been made to apply this technique to polysaccharides and fucoidans.

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO) (Burton, 1994; Fox, 1991; Robinson & Eskin, 1991; Sanchez-Amat & Solano, 1997; Wong, 1995), is a copper containing mixed-function oxidase widely distributed in microorganisms, animals and plants. This oxidase catalyzes two distinct reactions of melanin synthesis, the hydroxylation of a monophenol and the conversion of an *o*-diphenol to the corresponding *o*-quinone (López-Serrano, Solano, & Sanchez-Amat, 2004; Lontie, 1984; Swan, 1974). The hydroxylation of L-tyrosine, the initial step in melanin synthesis, is of considerable importance since it is also the initial step in catecholamine synthesis. Alterations in melanin synthesis occur in many disease states. Melanoma specific anticarcinogenic activity is also known being linked with tyrosinase activity (Prezioso, Epperly, Wang, & Bloomer, 1992). Melanins are also found in the mammalian eye and brain. Tyrosinase may play a

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role in neuromelanin formation in the human brain and could be central to dopamine neurotoxicity as well as contribute to the neurodegeneration associated with Parkinson's disease (Xu et al., 1997). Tyrosinase is also responsible for browning in plants and considered to be deleterious to the color quality of plant derived foods and beverages. This unfavorable darkening from enzymatic oxidation generally results in a loss of nutritional and economic values and has been of great concern (Friedman, 1996; Jang, Sanada, Ushio, Tanaka, & Ohshima, 2002; Lin, Qian, & Yang, 2010). Similarly, the unfavorable browning caused by tyrosinase on the surface of seafood products has also been of great concern (Kajiwara, Matsui, Akakabe, Murakawa, & Arai, 2006; Kamkaen, Mulsri, & Treesak, 2007; Ogawa, Perdigao, Santiago, & Kozima, 1984). Therefore, the regulation of the tyrosinase activity by its inhibitors has been the hot topic in recent ten years due to its potential applications in medicine, cosmetics and agriculture. To date, many different tyrosinase inhibitors have been suggested as being potential candidates for whitening agents or bio-insecticides (Huang et al., 2009; Kim & Uyama, 2005; Kubo, Kinst-Hori, & Yokokawa, 1994; Nerya et al., 2003). Thus, it is interesting to investigate if kelp fucoidan or polysaccharide can become a potential tyrosinase inhibitor for the above-mentioned use.

In the present study, HSCCC in combination with DEAE-Sepharose fast flow (F.F.) anion-exchange chromatography was used to purify a fucoidan from kelp polysaccharide. GPC–HPLC and FT-IR spectrum analysis was performed to analyze the characterization of the purified fucoidan. The inhibitory kinetics of the fucoidan for tyrosinase toward L-tyrosine was also investigated.

2. Materials and methods

2.1. Reagents and materials

Kelp polysaccharide was prepared according to our previously reported method (Yu & Chao, 2013) and was kept in our laboratory until use. PEG1000, L-tyrosine and the fucoidan standard sample were purchased from Sigma Co. Ltd. All other reagents were of analytical grade.

2.2. Preparation of the HSCCC sample

Kelp polysaccharide was deproteinated by combining TCA with the savage method (Song, Li, & Liu, 2009), and was exhaustively dialysed against water for 48 h. The concentrated dialysate was precipitated with four volumes of absolute ethanol. The precipitate was then washed with absolute ethanol and collected as the refined polysaccharide. The HSCCC sample was prepared by dissolving 1.0 g of the refined polysaccharide into 50 mL of the lower phase of a two-phase solvent system composed of PEG1000–K_2HPO_4–KH_2PO_4–H_2O (0.5:1.25:1.25:7.0, w/w).

2.3. Selection of the solvent system

The first step before performing a separation is to choose a suitable two-phase solvent system by measuring the partition coefficient of the particular target compound. To obtain a suitable solvent system for HSCCC, the partition coefficient K of the sample was detected by TLC. The suitable partition coefficient for HSCCC is approximately $K = 1$, and the sample is soluble in both the lighter and denser phase of the two-phase solvent system (Schwarz, Hillebrand, Habben, Degenhardt, & Winterhalter, 2003). To identify a suitable solvent system, two-phase solvent mixtures composed of PEG1000, K_2HPO_4, KH_2PO_4 and water in different ratios were investigated, and the partition coefficient (K) was determined by TLC. First, 0.5 mg of the refined polysaccharide and PEG1000–K_2HPO_4–KH_2PO_4–H_2O in different ratios were added to a 10 mL test tube with a cap. The sealed tube was shaken vigorously for several minutes to allow complete equilibration. After two clear layers formed, TLC analysis of the polysaccharide components in the two phases was performed. The concentration of polysaccharide components in the two phases was estimated by the color reaction. The partition coefficient for each polysaccharide component in the two phases was calculated.

2.4. HSCCC procedure

HSCCC experiments were performed with a two-phase solvent system composed of PEG1000–K_2HPO_4–KH_2PO_4–H_2O in a ratio of 0.5:1.25:1.25:7.0 (w/w). The upper phase was used as the stationary phase and the lower phase as the mobile phase. The multilayer coiled column was first filled with the upper phase, and the lower phase was pumped into the inlet of the column using a Waters 510 HPLC pump (Millipore Corporation, Milford, MA, United States) at a flow rate of 2.0 mL/min in a head-to-tail elution mode. The apparatus was rotated at 600 rpm. When the front of the mobile phase eluted from the outlet of the column, 1 g of the refined polysaccharide dissolved in 50 mL of the lower phase was injected into the preparative HSCCC system, and the effluent was collected with a BS-100 mode fraction collector (Shanghai Instrument Factory, Shanghai, China). Next, 10 mL of each fraction was subjected to TLC analysis. Fractions comprised of the same polysaccharide component were combined and dialysed with Visking dialysis tubes (molecular weight cut-off of 14,000 Da) to remove the salt and PEG. The resultant polysaccharide component was evaporated under vacuum conditions and freeze-dried.

2.5. Thin-layer chromatography (TLC) analysis of HSCCC fractions

The identity and purity of the obtained HSCCC fractions were determined by TLC. The stationary phase was silica gel 60 F254 from Merck (Darmstadt, Germany), and the mobile phase was *n*-butanol–ethanol–water (5:4:6). After development, the TLC plates were dried and sprayed with an aniline-diphenylamine solution. Blue spots were visible.

2.6. Purification of the KPS-2 fraction

For purification, 50 mg of the KPS-2 fraction was dissolved in 2 mL of distilled water and centrifuged at 10,000 rpm for 10 min. The supernatant was loaded onto a DEAE-Sepharose F.F. anion-exchange column (2.6 cm × 37 cm) pre-equilibrated with distilled water. After the column was equilibrated with the distilled water, a stepwise elution with increasing concentrations of sodium chloride solution (from 0 to 2 M) was carried out at a flow rate of 1.25 mL/min. The eluate was collected using a BS-100 mode fraction collector (Shanghai Instrument Factory, Shanghai, China). The total polysaccharide content in each tube was measured at 490 nm using the phenol–sulphuric acid colourimetric method.

2.7. Homogeneity analysis

Gel-permeation chromatography (GPC) has been shown to be an effective method for the homogeneity determination of the polysaccharide (Lopez-Barajas, Lopez-Tamames, & Buxaderas, 1998). The homogeneity of KPS-2-1, KPS-2-2 and KPS-2-3 was determined by GPC–HPLC. The chromatographic conditions are as follows: Waters HPLC ultrahydrogel linear column (7.8 mm × 300 mm); column temperature, 30 °C; mobile phase, pure water; flow rate, 0.7 mL/min; refractive index detector; detection temperature, 40 °C.

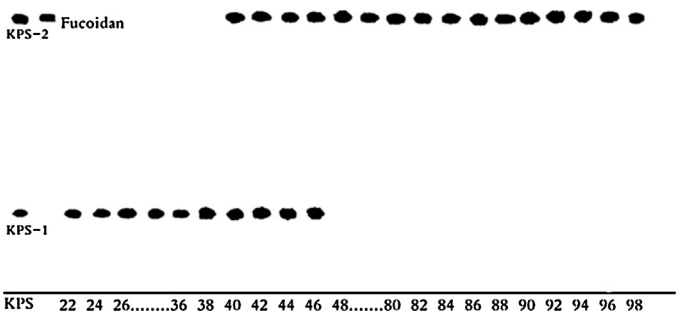

Fig. 1. Simulation map of the TLC analysis of fractions from HSCCC separation: fractions 22–46, KPS-1; fractions 48–98, KPS-2. TLC was developed using *n*-butanol–ethanol–water (5:4:6, v/v), and separated samples were colored with aniline-diphenylamine at 110 °C.

2.8. FT-IR analysis

Fourier transform infrared spectroscopy (FT-IR) was used to investigate the vibrations of the molecules and polar bonds among different atoms. The structures of the purified fucoidan were analyzed using FT-IR spectroscopy. To this end, 2 mg of the solid sample was analyzed on a Nicolet 380 spectrophotometer (Thermo Scientific, USA). The absorptive spectra were recorded on a Nicolet Impact 410 Fourier transform infrared spectrophotometer (Thermo Nicolet Co. Ltd., United States) in the range of 4000–400 cm^{-1}.

2.9. Inhibitory kinetics of fucoidan for tyrosinase

The tyrosinase activity was determined as described by Chang (2007) with minor modifications. First, 2.5 mL of 7.5 mM L-tyrosine dissolved in phosphate buffer (pH 6.8) was mixed with 0.5 mL of the purified fucoidan (dissolved in PBS) at 30 °C for 10 min. Next, 0.5 mL of the tyrosinase was added to initiate the reaction. The assay mixture was incubated at 30 °C for 3 min. The increase in the absorbance value at 475 nm due to the formation of the dopachrome was monitored using a spectrophotometer. One unit of the tyrosinase activity was defined as the amount of the enzyme needed to increase the absorbance value at 475 nm by 0.001 within 1 min under predefined conditions. The inhibitory percentage of the tyrosinase activity was calculated as follows:

$$\text{Inhibition percentage}(\%) = \frac{A - B}{A} \times 100 \quad (1)$$

where A is the absorbance at 475 nm with PBS as the control and B is the absorbance value at 475 nm with the sample. The concentration of the compound at which 50% of the enzyme activity was inhibited (the IC_{50} value) was obtained by linear fitting. The inhibitory kinetics of fucoidan for tyrosinase was analyzed using the Lineweaver–Burk method.

3. Results and discussion

3.1. Solvent system for the separation of polysaccharide components

HSCCC is a liquid–liquid partition chromatography approach in which separation is based on the difference in the partition coefficient (K) of solutes within a two-phase solvent system. The selection of the solvent system is important for HSCCC because it is equivalent to choosing both the column and the eluent. Successful separation by HSCCC largely relies on the selection of a suitable two-phase solvent system. The partition coefficient (K),

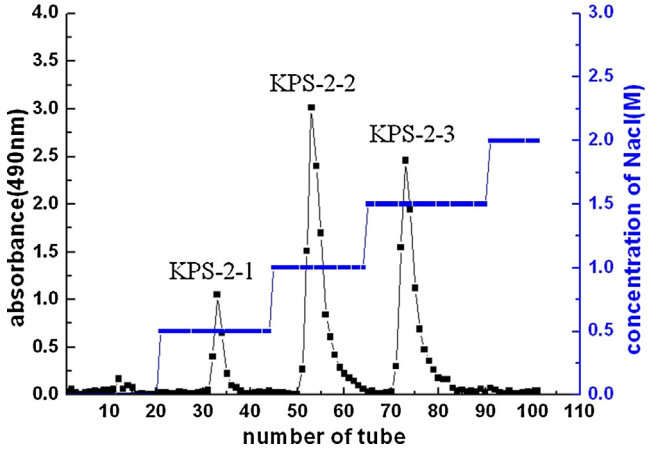

Fig. 2. Stepwise elution curve of KPS-2 on a DEAE-Sepharose F.F. anion-exchange chromatography column. KPS-2-1, KPS-2-2 and KPS-2-3 represent three fractions from KPS-2 obtained from kelp polysaccharide. NaCl concentrations used are 0–2 M and the flow-rate of the NaCl solution is 1.25 mL/min. The effluent was monitored at 490 nm by the phenol–sulphuric acid colourimetric method. The NaCl concentration corresponding to each fraction is presented in blue. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

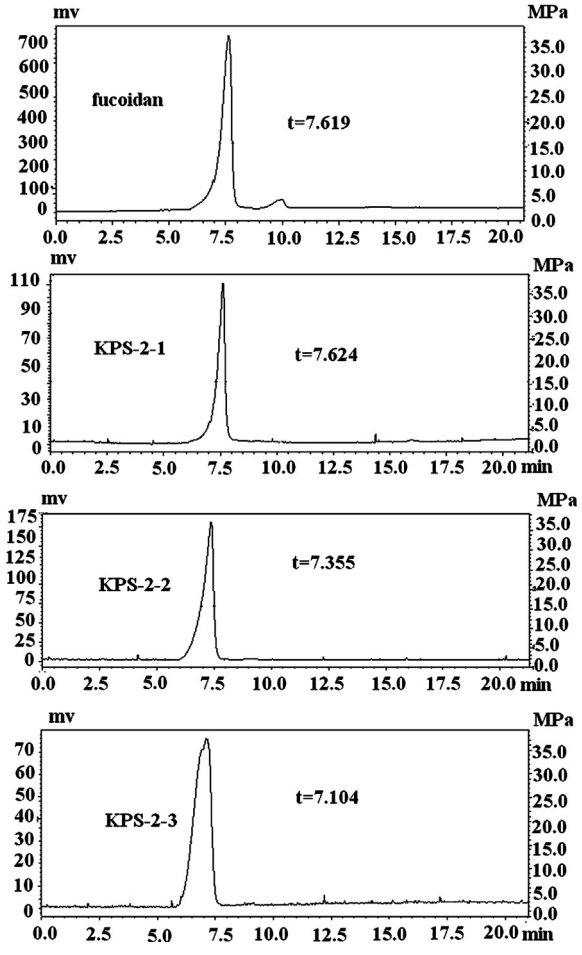

Fig. 3. GPC–HPLC profiles of KPS-2-1, KPS-2-2 and KPS-2-3 from KPS-2. Signals of the fucoidan sample (7.619 min), KPS-2-1 (7.624 min), KPS-2-2 (7.355 min) and KPS-2-3 (7.104 min) are indicated in the chromatograms. KPS-2-1 has the same retention time as the standard sample. Chromatographic conditions are as follows: Waters HPLC ultrahydrogel linear column (7.8 mm × 300 mm); column temperature, 30 °C; mobile phase, pure water; flow rate, 0.7 mL/min; refractive index detector; detection temperature, 40 °C.

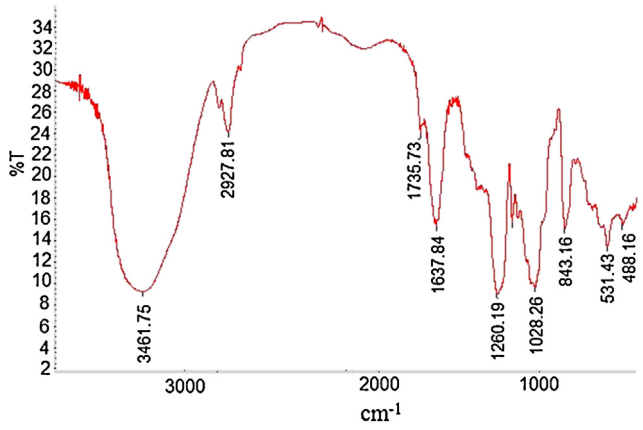

Fig. 4. Fourier transform infrared spectroscopy analysis of the KPS-2-1 fraction.

generally within the range of 0.4–2.5, is an important parameter for HSCCC separation (Duarte, Cardoso, Noseda, & Cerezo, 2001). A lower K value means that the separated solute is closer to the solvent front with a lower resolution, whereas a larger K value tends to give a better resolution but broader, more diluted peaks due to the longer elution time. To achieve an efficient separation of polysaccharide components in a high-molecular weight complex, a large difference in the partition coefficients of the isolated components is essential. In this study, TLC was used to check the partition of polysaccharide components between two phases of a series of solvent systems composed of PEG1000 and potassium phosphate solutions in different proportions. The K value was calculated as the ratio of the concentration of each polysaccharide component in the upper phase to the concentration in the lower phase. The solvent system PEG1000–KH_2PO_4–K_2HPO_4–water with the ratio of 0.5:1.25:1.25:7.0 (w/w) gave K values of 0.5 and 1, which was suitable for the separation of target compounds from kelp polysaccharide by HSCCC.

3.2. HSCCC separation

The result of the HSCCC separation is shown in Fig. 1. The sample solution contained 1.0 g of the polysaccharide and 50 mL of the lower phase of the two-phase solvent system composed of PEG1000–K_2HPO_4–KH_2PO_4–H_2O in the ratio of 0.5:1.25:1.25:7.0 (w/w). The separation was performed at 600 rpm at a flow rate of 2.0 mL/min using the lower phase as the mobile phase. The TLC analysis of separated HSCCC fractions gave blue spots at $R_f \sim 0.3$ in fractions 22–46 and $R_f \sim 0.9$ in fractions 40–98. Fractions 48–98 corresponded to polysaccharide components containing fucoidan, and parts of them showed the same R_f value as the fucoidan standard sample. Fractions 48–98 were combined and dialysed to remove the salt and PEG. The resultant product was evaporated under vacuum and then freeze-dried to obtain the polysaccharide component containing fucoidan (KPS-2).

3.3. Purification of KPS-2

The KPS-2 fraction was purified by DEAE-Sepharose F.F. anion-exchange chromatography, yielding three independent elution peaks, as detected by phenol–sulphuric acid assay (Fig. 2). Each fraction of KPS-2 was concentrated and dialysed to give KPS-2-1, KPS-2-2 and KPS-2-3. The GPC–HPLC profiles of KPS-2-1, KPS-2-2, KPS-2-3 and the fucoidan standard sample are shown in Fig. 3. It was obvious that KPS-2-1 had the same retention time as the fucoidan standard sample, and may be used for further analysis.

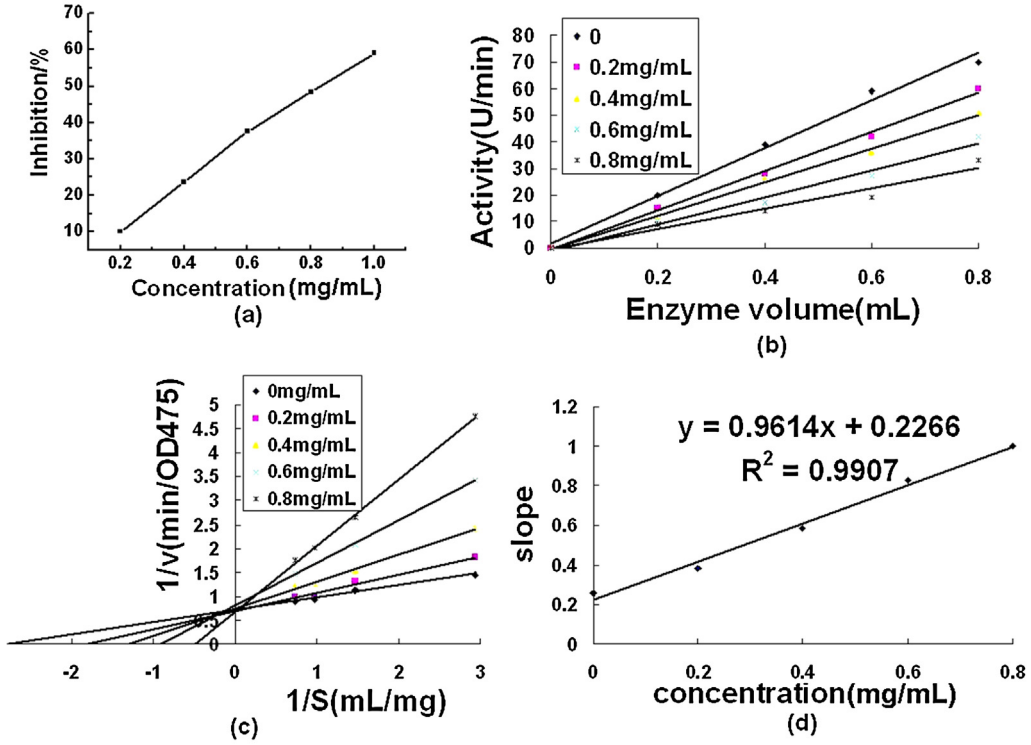

Fig. 5. (a) Inhibitory effect of the KPS-2-1 fraction on the tyrosinase activity toward L-tyrosine. (b) Relationship between the catalytic activity of tyrosinase and the enzyme volume at different concentrations of KPS-2-1. The L-tyrosine concentration is 7.5 mmol/L, and the reaction time is 3 min. (c) Lineweaver–Burk plots of the inhibition of KPS-2-1 for tyrosinase toward L-tyrosine. (d) Plots of the slope versus the concentration of KPS-2-1 to determine the inhibitory constant K_i.

3.4. FT-IR analysis of KPS-2-1

The FT-IR spectrum of KPS-2-1 is presented in Fig. 4. The primary absorptive peaks were characteristic of glycosidic structures, and were related to C—O stretching (1028.26 and 1268.26 cm^{-1}) and the vibration of the anomeric C—H group (845.16 cm^{-1}). Moreover, the characteristic absorptive peak at 845.16 cm^{-1} in the IR spectrum indicated that the anomeric C—H was in the α-configuration. The absorptive peak at 1260.19 cm^{-1} was assigned to the stretching of the S—O bond. These results indicate that KPS-2-1 is a sulphated polysaccharide. The FT-IR spectrum of KPS-2-1 exhibited an intense and broad peak at 3461.75 cm^{-1}, which was a hydroxyl stretching vibration. The peak at 2927.81 cm^{-1} corresponded to the stretching vibration of the weak C—H bond.

3.5. Inhibitory kinetics of fucoidan for tyrosinase

The effect of different concentrations of fucoidan (from 0.2 mg/mL to 1.0 mg/mL) on the oxidation of L-tyrosine by tyrosinase was studied, and the result is shown in Fig. 5(a). With the increase in the concentration of fucoidan, the activity of tyrosinase decreased significantly. At 1.0 mg/mL fucoidan, the activity of tyrosinase was inhibited by 62.21%. The IC_{50} value of the fucoidan for tyrosinase inhibition was determined to be 0.82 mg/mL. Fig. 5(b) shows the relationship between the catalytic activity of tyrosinase and the enzyme dosage under different concentrations of fucoidan. Plots of the activity of the residual enzyme versus the volume of enzyme used in the presence of different concentrations of fucoidan gave a family of straight lines, all of which passed through the origin. Increasing the fucoidan concentration resulted in a decrease in the line slope, indicating that the inhibition of tyrosinase by fucoidan was reversible. The presence of fucoidan did not reduce the amount of the enzyme; it merely inhibited the enzyme activity. The inhibitory kinetics for tyrosinase by fucoidan is illustrated in Fig. 5(c). Under the experimental conditions employed, the oxidation of L-tyrosine followed the Michaelis–Menten equation. Double-reciprocal plots gave a family of straight lines intersecting in the 3rd quadrant. As the fucoidan concentration increased, K_m increased and V_m remained constant. Thus, fucoidan was a competitive inhibitor of tyrosinase. The inhibitory constant K_i obtained from the double-reciprocal plots shown in Fig. 5(d) was 0.9907 mg/mL.

4. Conclusions

The results of this study demonstrate that HSCCC with sequential DEAE-Sepharose F.F. anion-exchange chromatography is an effective method for the purification of the fucoidan from kelp polysaccharide. This method provided a purified compound KPS-2-1, identified as a sulphated fucoidan by GPC–HPLC and FT-IR analysis. The purified fucoidan was demonstrated to be a strong competitive inhibitor of the tyrosinase toward L-tyrosine. This study provides a useful foundation for the future use of kelp polysaccharide in the fields of medicine, cosmetics and agriculture.

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